Modified substrates of DNA polymerases and design of antivirals

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The results obtained in our laboratory on investigating of substrate properties of a large number of compounds towards different DNA polymerases have been summarized. On the basis of systematic analysis a directed synthesis of nucleotides with antiviral properties was performed.

Rational search for inhibitors of human immunodeficiency virus (HIV) reproduction in a series of nucleosides and nucleotides has been performed according to some empirical rules developed in several laboratories, including ours, during the last decade. The requirements concerning these compounds were chosen as follows. Deoxynucleosides or nucleotides must penetrate into HIV or other virus infected cells, be phosphorylated to corresponding modified deoxynucleoside 5'-triphosphates (dNTP) by cellular enzymes with subsequent effective and selective inhibition of DNA synthesis catalyzed by HIV reverse transcriptase on natural RNA and DNA templates, or by DNA polymerases of other human viruses. In this review we do not take into account some essential effects which such compounds exert on the rate of DNA metabolism in human organisms, the rate of formation of viral types resistant to the action of these compounds, etc. These aspects can be discussed elsewhere. A few deoxynucleosides and nucleotides, however, are known to possess the above mentioned properties.

Our research dealt mainly, in a comparative aspect, with the behavior of human DNA polymerases and viral reverse transcriptases, including HIV reverse transcriptases, towards modified substrates as well as with evaluation of the contribution of sugar moiety (glycone) and phosphate modifications in nucleotides and nucleoside 5'-triphosphates to their substrate properties.

The results of comparative analysis of the role of glycone modifications in nucleoside 5'-triphosphates in the binding of the latter with DNA polymerases were described in previous publications [1-3]. This investigation was performed in collaboration with Dr. R. Bebealashvilli group (National Cardiology Research Center, Moscow), and Dr. J. Grulman group (American Cyanamid Company, Pearl River, U.S.A.). Herein we present only the main conclusions of this part of the research.

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Abbreviations: CMV, cytomegalovirus; dNTP, deoxynucleoside 5'-triphosphates; HSV-1, herpes simplex virus type 1; TdT, terminal deoxynucleotidyl transferase.
1. Retroviral reverse transcriptases are less specific towards glycone modifications in \( \beta\)-\( \beta\)-nucleoside 5'-triposphates than are human DNA polymerases.

2. Modified 2'-deoxynucleoside 5'-triposphates with bulky substituents at 3'-position (N\(_3\) [4], NHR, N\(_2\)R [5], SH [6], NO\(_2\) [7]) selectively inhibit, by a terminating mechanism, DNA synthesis catalyzed by retroviral reverse transcriptases.

3. Retroviral reverse transcriptases are also less specific towards conformationally restricted modified nucleoside 5'-triposphates [1, 8–12]. This was observed most clearly in the cases when the glycone structure could be made a planar one, with low energy consumption. We imply that the planar conformation of the glycone ring favors the transition state of a substrate in the DNA synthesizing complex.

4. Human replicative DNA polymerases \( \alpha \) and \( \varepsilon \) reveal the highest substrate specificity; the repairing DNA polymerase \( \beta \) and terminal deoxynucleotidyl transferase show lower specificity.

5. Substrate specificity of the herpes simplex virus type 1 (HSV-1), cytomegalovirus (CMV), and adenovirus DNA polymerases is very close to that of human replicative DNA polymerases but it is slightly lower.

It should be mentioned that human DNA polymerases \( \delta \) and \( \gamma \) were not at our disposal. Therefore we could not compare their substrate specificity with that of viral enzymes.

Studies on the influence of triposphate modifications in nucleoside 5'-triposphates on their substrate properties demonstrated that the introduction of additional methylene group at 5'-position of triposphate (I, in Scheme 1) resulting in the hydrolytically stable P–C bond does not prevent the incorporation of the modified compound into the growing DNA chain in the process of DNA synthesis catalyzed by HIV reverse transcriptases [13]. If \( \beta\gamma\)-diphosphate residue of nucleoside 5'-triposphates is substituted by dibromomethylene phosphonic, difluoromethylene phosphonic or phosphonoacetate groups [13, 14] the affinity of such compounds towards DNA polymerases is strongly decreased whereas their affinity towards HIV reverse transcriptases is practically retained. Similar results were obtained for I towards HIV reverse transcriptases [15].

Hydroxyl substitution at \( \omega \)-phosphate residue by a methyl [16] or a phenyl group [17] (compounds II, in Scheme 1) also reduces substrate properties of nucleoside 5'-triposphates towards retroviral reverse transcriptases but to a much lesser extent than towards human DNA polymerases.

\[ \text{Scheme 1} \]
We investigated the influence of β,γ-diphosphate replacement by other anionic residues. Compounds III (Scheme 1) were evaluated as terminating substrates towards retroviral reverse transcriptases. This part of the research was performed together with G. Galegov group (Institute of Virology, Moscow). The dNTP analogs in which β and γ phosphorous are bound through NH [18] or CH$_2$ [19] groups revealed only slightly reduced properties.

The results of these studies show that natural 2'.3'-deoxynucleoside 5'-triphosphates even when modified at the glycone or at any phosphorus atom, can retain their substrate properties. The limits for such modifications and their combinations are under investigation. This research could be of use both for the investigation of the structure of DNA polymerases active centers and for designing of antiviral, antibacterial, antifungal, and anticancer drugs.

Then we evaluated a number of 2'.3'-deoxynucleoside 5'-phosphonates (IV) as potential antiviral agents (Scheme 2). We expected that these compounds, if phosphorylated by cellular enzymes to corresponding modified triphosphates III (where R' = H, Me or an other radical), could selectively inhibit HIV replication. The tested compounds proved, however, to be inactive with the exception of IV (B = Gua, R = COOH) that inhibited HIV replication by 50% at a concentration of 5 μM [20] in MT-4 cell culture. The lack of activity of the other compounds could have resulted from lack of recognition of these phosphonates by cellular phosphorylating enzymes. Other reasons are also possible, for instance, hydrolysis of compounds IV by dephosphorylating enzymes to natural deoxynucleosides. The latter, which undergo intensive phosphorylation thus compensating the inhibiting effect of the corre-
sponding pyrophosphorylphosphonates that might be formed from IV. To check this meta-
bo lic pathway we tested the influence of IV (R = H) on inhibition of HIV replication by
AZT. If IV (R = H) is hydrolyzed in the cells (even partially) it would compensate AZT ac-
tion by the forming thymidine and in fact, H-
phosphonate IV (R = H) completely com-
pen sated the inhibition effect of AZT that favors the enzymatic hydrolysis of IV (R = H) [20].

Then we evaluated hydrolysis bearing two modifications — at glycone and at 5'-phos-
ph ate residues. Among them there were 3'-modi fied 2',3'-dideoxynucleotides (V) [21-27],
2',3'-dideoxy-2',3'-dideoxynucleotides (VI)
[24], 2',3'-anhydrocyclonucleotides of ribo- and
lyxo-series (VII, VIII) [24], ribonucleotide ace-
tals, ketals and orthoesters (IX) [28] (Scheme 2).

Several highly active inhibitors of HIV replication in some cell cultures were found, espe-
cially Va (B = Thy, R = H, F, CH₂OH, COOH,
CH₂Cl, COOEt, R' = N₃); Vb (B = Thy, R = F,
R' = F); Vc (B = Ade, R = H, R', R' = H); Vd
(B = Ade, R = H, R' = H, F); Ve (B = Gua,
R = R' = H); VI (B = Thy, R = F); IX (B = Ade,
R = H, R' = OMe) should be mentioned. Some
other nucleoside 5'-phosphonates of V, VI, IX
showed moderate activity, which, as a rule,
was lower than that of the parent nucleo-
sides. Many compounds were inactive. Sub-
stances X revealed no activity [29] either.
Antiviral researches were performed in Dr C.
Galegov and Dr E. Karamov groups, Institute
of Virology, Moscow, in Department of Prof. Dr
E. De Cicerq (Katolike University, Leuven), in
the groups of Drs K.A. Watanabe and Dr B.W.
Polsky (Memorial Sloan Kettering Cancer Cen-
ter, New York), and the group of Dr I. Gluzman
(Am erican Cyanamid Company, Pearl River).
None of the compounds were reasonably active as inhibitors of human HSV-1 and 2 types, CMV
or adenovirus.

The interpretation of the above data is diffi-
cult. One can mention that the activity values
within the series of phosphonates of different
3'-modified nucleosides do not correlate. For
example, antiviral activity values in the series
of 3'-fluoro-3'-deoxythymidine 5'-phospho-
notes differ from those for the same phospho-
notes of 3'-azido-2',3'-dideoxythymidine.

The metabolism of nucleoside 5'-phos-
phonates is very poorly known. We examined sta-
ibility of the most interesting compounds both
in the presence of dephosphorylating enzymes and in human blood serum [30, 31].
Phosphonates IV and V (B = Thy, R = H) were shown to be resistant to dephosphorylation
even after five days incubation with Escherichia
coli alkaline phosphatase or phosphatases from
human placenta and calf intestines. However,
snake venom 5'-nucleotidase slowly dephos-
phorylated most of these compounds. The dephosphorylation rate for IV, V (R = H) was 5–7
and 0.3%, respectively, of that for thymidine
5'-phosphate. Under the same conditions, the
dephosphorylation rate for V (B = Thy) depended
on the structures of both R and R', the final
effect being a combination of their separate
effects. The hydrolysis of IV (B = Thy, R = H or
F), and V (R' = For N₃, R = H) in human serum
was also reduced. For example, the dephos-
phorylation rate for IV and V with R = H, reached
only 4–7% of the rate for the corresponding
5'-phosphates, whereas the dephosphorylation rate for IV and V with R = F decreased only by
a half. The reaction ran in two stages, replace-
ment of the fluorine by the hydroxyl followed
by hydrolysis of the phosphate group. Com-
ounds IV and V (B = Ade) with R = H, COOH,
Me, R' = H, OH were shown to be stable in
human serum even after a week at 37°C; neither
deamination nor apurinization were observed.

All these data demonstrate that the metabo-
lism rate of nucleotides modified at the phos-
phate or both phosphate and glycone residues
decreases as compared with that for nonmodi-
fied nucleotides and depends on the nature of
these modifications and their combination.
Probably slower metabolic transformations of
nucleoside 5'-phosphonates in comparison
with the corresponding nucleosides can ac-
count for the low toxicity of the former in cell
cultures.

Taken together, these data demonstrate that
nucleoside 5'-phosphonates are involved, due
to the wide range of their properties both in
inhibition of HIV replication and in cellular
metabolism. The same picture is observed for
different nucleosides modified at the glycone.

We come back now to modified nucleoside
5'-triphosphates. Comparative investigation
on substrate specificity of different DNA
polymerases towards various modified nucleo-
side 5'-triphosphates with terminating and
other modes of action, resulted in preparation of
several compounds specifically inhibiting
DNA synthesis catalyzed by different types of these enzymes.

As it was mentioned above, several highly specific terminating substrates of reverse transcriptases of retroviruses are known at present. Modified substrates I (R = OH) were shown to be specific for replicative DNA polymerase α [32]. The nucleotide residue of this compound is incorporated into 3'-terminus of DNA, sharply decreasing the rate of chain elongation and practically terminating the synthesis. Triphosphate I (R = OII) demonstrates the same properties towards DNA polymerases of HSV-1 and CMV [33, 34].

Arabino nucleoside 5'-triphosphates with a bulky 2'-substituent proved to be highly specific inhibitors of human HSV and CMV DNA polymerases [35]. They are not recognized by human DNA polymerases and retroviral reverse transcriptases. Nucleotide residues of these compounds are incorporated into DNA chain in the process of DNA biosynthesis catalyzed by viral DNA polymerases and stop further elongation. Arabino compounds with NH₂ and N₃ at the 2'-position reveal substrate properties towards both viral and human DNA polymerases and, after incorporation into growing DNA, terminate chain elongation [33–35].

So far, no highly selective inhibitors of terminal deoxynucleotidyl transferase (TdT) are known. This may be one of the reasons of the lack of data on its function in the cell. A high concentration of this enzyme was observed in premature leukocytes and in the cells of some forms of leukemia. We have found that modified nucleoside 5'-triphosphates with the trans-like position of the nucleic base towards 5'-CH₂-group are not recognized by template-dependent DNA polymerases but terminate DNA synthesis catalyzed by TdT [36]. Probably this rule is not a general one, but there are some other trans-like dNTP shown to be specific inhibitors of TdT [36].

Our results together with those from other laboratories allowed us to initiate the construction of DNA polymerase active center models. Two methods can be used for this purpose and within certain limits they are complemental. In the first one the necessity of functional groups in 2'-deoxynucleotide 5'-triphosphates (dNTP) substrates for their interaction with complexes (DNA polymerase + template-primer) is ana-

lyzed. The direct interaction of glycone functional groups in dNTP with the enzyme by formation of ionic or hydrogen bonds was shown not to be critical. The data available allow to conclude that practically no part of the glycone residue has any fatal influence on substrate properties of the dNTP modified in such a way towards different DNA polymerases including reverse transcriptases [37]. The glycone seems to play the role of a template only. It has to orientate properly the nucleic base and the triphosphate residue. However, these results should be interpreted with caution because of two reasons. The first is the wide diversity of conditions used by different researchers. This concerns especially the structure of template-primer complexes (monotonous poly(rA)-oligo(dT), poly(dA)-oligo(dT), and alternating poly[d(A-T)] and others). The scatter of the results obtained in such artificial systems is too wide. For example, Michaelis constants for the same modified dNTP differed in various systems up to two orders of magnitude. Secondly, for serious generalizations the quantitative measurements of affinity of modified substrates to DNA, synthesized complexes are required. In our opinion in such studies only template-primers with all four natural nucleotides should be used and only under standardized conditions.

In the second method, modified dNTP with bulky substituents at different positions of the molecule are used. The incorporation of such dNTP into DNA chain can mean that there are no topological restrictions for the site of this substituent. Several such positions in a substrate molecule are found. Some of them, namely 5'-position of a pyrimidine and 7-position of a purine in dNTP are common for all DNA polymerases studied including E. coli DNA polymerase I [38, 39], T4 phage enzyme, α and βDNA polymerases of human origin, retroviral reverse transcriptases, DNA polymerases of human DNA containing viruses and other enzymes [40–42]. Some other substitutions are rather uncommon. As it was mentioned before CMV and HSV DNA polymerases are able to incorporate the nucleotide residue of arabino compound into DNA 3'-termini. One of the stereoisomers of compound III has been shown to be a substrate of some DNA polymerases. Some other dNTP with bulky substituents are also known to be substrates of different DNA.
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